Protoplast-Based Transient Expression Combined with Plant Cultivation Systems as a Valuable Tool for Floral Thermogenesis Studies in Aroids

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Research Article

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Abstract

Floral thermogenesis in plants plays a significant role in their reproductive function. Thermogenic aroids constitute a large family in highly thermogenic angiosperms, many of which possess intense heat-producing abilities. Several genes have been proposed to be involved in floral thermogenesis of aroids, but the biological tools to identify the functions of those genes at cellular and molecular levels are lacking. Among the many thermogenic aroids, we focused on skunk cabbage (*Symplocarpus renifolius*) because of its ability to produce intense, durable heat and small aboveground parts compared with other thermogenic aroids. In this study, leaf protoplasts were isolated from potted and shoot tip-cultured skunk cabbage plants and used to develop transient assay systems. The isolation protocol included an additional, sucrose gradient centrifugation step, which yielded high-purity protoplasts from both types of plants. The isolation and transfection efficiency of the protoplasts exceeded 1.0×10^5/g fresh weight and 50%, respectively, in both potted and shoot tip-cultured plants. Using this protoplast-based transient expression (PTE) system, we determined the protein localization of three mitochondrial energy-dissipating proteins, SrAOX, SrUCPA, and SrNDA1, fused to green fluorescent protein (GFP). In skunk cabbage leaf protoplasts, these three GFP-fused proteins were localized in MitoTracker-stained mitochondria. However, the green fluorescent particles in protoplasts expressing SrUCPA-GFP were enlarged compared with those in protoplasts expressing SrAOX-GFP and SrNDA1-GFP. Our PTE system is a powerful tool for functional gene analysis not only in thermogenic aroids but also in non-thermogenic aroids.

Introduction

Plants with heat-producing ability in their reproductive organs (inflorescences, flowers, and cones) are widely distributed among gymnosperms and angiosperms (Gibernau et al. 2005; Seymour 2010; Tang 1987). Increased temperature in their flowers or cones enables them to effectively diffuse volatile odor components (VOCs) as attractants, which renders this ability to produce heat, also known as thermogenesis, an important reproductive strategy to attract pollinators (Meeuse and Raskin 1988). The principal composition of VOCs emitted from the inflorescences was revealed in several thermogenic species, such as aroids (Kite and Hetterscheid 2017; Marotz-Clausen et al. 2018; Oguri et al. 2019; Shirasu et al. 2010; Stensmyr et al. 2002) and cycads (Azuma and Kono 2006; Salzman et al. 2020; Terry et al. 2007). In the cycad *Macrozamia lucida*, specific pollinator thrips (*Cycadothrips chadwickii*) are attracted (“pulled”) by lower concentrations of the dominant VOCs, b-myrcene, and repelled (“pushed”) by higher concentrations of the same compound (Terry et al. 2007). This strategy is called “push-pull pollination” and is also used by another cycad *Zamia furfuracea*, which regulates the concentration of 1,3-octadiene to attract or repel its specific pollinator weevils (*Rhopalotria furfuracea*) (Salzman et al. 2020). In the two cycad species, volatile production is regulated by a daily thermogenic event, and owing to this strategy, the dioecious cycads receive the pollination service from their specific pollinators, which transfer the pollen from the male cone to the female cone (Salzman et al. 2020; Terry et al. 2007). Warm temperature in flowers or cones may also benefit insect pollinators by enhancing larval development, stimulating mating, and saving the energy for insect departure from flowers or cones (Seymour et al. 2003b; Terry et al. 2004, 2007).

Generally, thermogenic plants are defined as species that can increase their floral or cone temperatures to at least 0.5 °C above ambient temperature. On the basis of this definition, at least 80 plant species are considered thermogenic, half of which are gymnosperm cycads (Ito-Inaba et al. 2019; Tang 1987), and the remaining are

angiosperms comprising 36 species of aroids (Table 1) and a few species of Nymphaeaceae, Magnoliaceae, and Nelumbonaceae (Seymour 2010; Seymour and Matthews 2006). Many aroid species are with high heat-producing ability, and 7 species, namely giant taro (*Alocasia macrorrhizos*), arum lily (*Arum dioscoridis*), *Colocasia gigantea*, philodendron (*Philodendron selloum*), skunk cabbage (*Symplocarpus foetidus* and *S. renifolius*), and *Homalomenapendula*, can increase the temperature in flowers (spadix, appendix, or male florets) at least 15 °C above ambient temperature (Table 1). Furthermore, several species of philodendron and skunk cabbage are both thermogenic and thermoregulatory. Owing to the intense heat-producing ability and the large number of thermogenic species among aroids, this group of plants is very important in comprehensive studies on plant thermogenesis.

Among thermogenic aroids, Asian (*S. renifolius*) and American skunk cabbage (*S. foetidus*) possess thermogenic and thermoregulatory features—they can raise floral temperature in the spadix more than 20 °C above ambient temperature and keep it constant for about one week (Knutson 1974; Seymour and Blaylock 1999; Uemura et al. 1993). Although several processes may be important for thermogenic and thermoregulatory features in skunk cabbage, one of the principal processes is the short-term mechanism that depends on increased cellular respiration facilitated by mitochondrial energy-dissipating proteins, such as alternative oxidase (AOX), uncoupling protein (UCP), or type II NAD(P)H dehydrogenase (NDs) (Moore et al. 2013; Smith et al. 2004; Sweetman et al. 2019). AOX, as a terminal oxidase of the mitochondrial electron transport chain, transfers the electrons extracted from the electron pool ‘ubiquinone’ to oxygen without generating proton motive force and reduces oxygen into water. In many thermogenic tissues, levels of transcriptional expression and protein accumulation of AOX are observed at a high level. UCP dissipates proton concentration gradient formed through the inner mitochondrial membrane. In mammalian brown adipose tissues, mice lacking UCP1 are not able to keep internal temperature under cold condition. NDs transfers the electrons extracted from NAD(P)H to ‘ubiquinone’, and thus NDs may, together with AOX, form the nonphosphorylating pathway of electron transport, which is not coupled to ATP synthesis. However, the molecular tools for elucidating the mechanisms by which the three energy-dissipating proteins are involved in floral thermogenesis and thermoregulation have yet to be established.

Protoplasts have been isolated from leaf tissues of many plants, such as Arabidopsis (Yoo et al. 2007; Zhai et al. 2009), tobacco (Nagata and Takebe 1971), maize (Cao et al. 2014), rice (Fujikawa et al. 2014; Page et al. 2019; Zhang et al. 2011), and other non-model plant species (Burris et al. 2016; Sasamoto and Ashihara 2014). These protoplasts were used to develop an efficient protoplast-based transient expression (PTE) system that provides a great platform for gene function identification in numerous species from model to non-model plants. The PTE system has been widely used for protein localization, protein-protein interactions, and identification of gene function and gene regulation (Fujikawa et al. 2014; Lin et al. 2018; Page et al. 2019; Ren et al. 2020; Zhai et al. 2009). In addition, high-throughput techniques based on this system were established for analyzing transcription factors in Arabidopsis (Wehner et al. 2011) or protein localization in rice (Page et al. 2019). The protoplast can potentially develop into a whole plant under suitable conditions (Maćkowska et al. 2014; Nagata and Takebe 1971), and it was exploited to develop transgenic plants with non-pathogen-related methods when combined with genome-editing and gene silencing technologies. Adequate protoplast isolation methods and an efficient PTE system are lacking for aroid plants, and the shortage of genetic tools, including PTE system, has hampered the progress of molecular biology in floral thermogenesis.

In this study, we established an efficient method for protoplast isolation and transient gene expression using potted and shoot tip-cultured skunk cabbage plants. Using PTE systems in both potted and cultured plants, we determined the localization of three energy-dissipating proteins fused to the green fluorescent protein (GFP; SrAOX-GFP, SrUCPA-GFP, and SrNDA1-GFP). Our developed PTE system using a thermogenic aroid, skunk cabbage, combined with plant cultivation systems may be a valuable tool for functional gene analysis in studies on floral thermogenesis in aroids.

Materials And Methods

Plant material and growth conditions

Skunk cabbage, *Symplocarpus renifolius*, plants with flower buds were transferred from an outdoor field in Aomori, Japan, to the laboratory at the end of autumn and cultured as potted plants. Some of the plants were maintained on a shoot tip culture system as previously described (Kitaura et al. 2008). Briefly, shoot apexes were cut and cultured on 1× Murashige and Skoog (MS) medium (3% w/v sucrose, 0.05% v/v plant preservative mixture, 1 mg/mL 6-benzyladenine [BA], 0.1 mg/mL 1-naphthylacetic acid [NAA], 0.35% w/v Gelrite, pH 5.8) under 16 h weak light (2800-4400 lux)/8 h dark conditions at 20 °C and 50% relative humidity in a growth chamber (LPH-240S; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). When the shoots reached 3-5 cm in height, they were transferred to new 1× MS media prepared without NAA and BA and cultured under the same conditions in the growth chamber.

Isolation of protoplasts from skunk cabbage leaves

Protoplasts were isolated from skunk cabbage (*S. renifolius*) leaves following the isolation protocol of the Arabidopsis mesophyll protoplast (Yoo et al. 2007; Zhai et al. 2009), with some modifications. Briefly, approximately 3 g of skunk cabbage leaves were sliced with a razor blade into 0.5-1 mm strips on 3MM filter papers, and soaked in 20 mL enzyme solution (10 mM $CaCl_2 \cdot 2H_2O$, 400 mM mannitol, 20 mM 2-[N-morpholino]ethanesulfonic acid [MES], 0.25% macerozyme, 1% cellulase, pH 5.6). After 17 h incubation at 22 °C in a container covered with a lid to avoid evaporation of the enzyme solution, the protoplasts were released from sliced leaves by shaking the container. The crude protoplast solution was filtered through one layer of Miracloth pre-wetted with W5 solution (154 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 5 mM glucose, 1.5 mM MES, pH 5.6) to remove leaf residues, and filtrates containing the protoplasts were centrifuged at 100 × *g* and 20 °C for 3 min. The supernatant was decanted and the pellet with protoplasts was re-suspended into 10 mL of W5 solution. The protoplast solution (10 mL) was layered on 20 mL of 21% (w/v) sucrose solution and centrifuged at 100 × *g* and 20 °C for 3 min. The interface fraction containing protoplasts was recovered and resuspended in 20 mL of W5 solution. After centrifugation at 100 × *g* and 20 °C for 3 min, precipitates were resuspended in 10 mL of W5 solution and incubated on ice for 30 min. The samples were centrifuged at 100 × *g* and 20 °C for 3 min and the precipitate was resuspended in 300 µL of MaMg solution (400 mM mannitol, 15 mM $MgCl_2 \cdot 6H_2O$, 5 mM MES, pH 5.6). The protoplasts were counted using a hemocytometer under an optical microscope (Axio Imager A2; Zeiss, Jena, Germany).

Construction of protoplast transfection vectors

Total RNA was extracted from female-stage spadices of *S. renifolius* using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized from 1 mg total RNA using a PrimeScript^{TM} RT reagent Kit (Takara Bio Inc., Otsu, Japan). The resulting cDNA was then used as template in subsequent reverse transcription-PCR (RT-PCR) with KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan), and *SrAOX*, *SrUCPA*, and *SrNDA1* genes were amplified using primer sets: mSrAOX_F1 and mSrAOX_R1 for *SrAOX*, mSrUCPA_F1 and mSrUCPA_R1 for *SrUCPA*, and mSrNDA1_F1 and mSrNDA1_R1 for *SrNDA1*. The GFP genes were amplified from pUC*35S*::*GFP* using PCR with KOD-Plus DNA polymerase (TOYOBO) and the primers GFP-F1 and mGFP-R1-rev1. To generate the *35S::SrAOX-GFP*, *35S::SrUCPA-GFP*, and *35S::SrNDA1-GFP* constructs, the two amplified PCR products were mixed and cloned into the *Xba*I site of p35S-Luc using an In-Fusion HD Cloning Kit w/Cloning Enhancer (Clontech Laboratories, Inc., Mountain View, CA, USA). The resulting DNA inserts were sequenced with the primers CaMV35S pro-90 and NOS term. All primers used in this study are summarized in Supplementary Table 1.

PEG-mediated transient expression in skunk cabbage leaf protoplasts

2×10^5 cells of protoplasts and 10 mg of DNA (*35S::GFP*, *35S::SrAOX-GFP*, *35S::SrUCPA-GFP*, *35S::SrNDA1-GFP*) were added to the MaMg solution and the volume was adjusted to 155 mL. The solution with protoplasts and DNA was mixed with 150 mL polyethylene glycol (PEG) solution (0.2 M mannitol, 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$, 40% (w/v) PEG4000) prepared just before use. The mixed solution was incubated at room temperature for 30 min to transfect DNA into the cells, and the reaction was stopped by adding 5 mL of W5 solution. Transfected protoplasts were precipitated by centrifugation at 50 × *g* and 20 °C for 3 min, and resuspended in 1 mL washing/incubation (WI) solution (0.5 mM mannitol, 20 mM KCl, 4 mM MES). Finally, the transfected protoplasts in WI solution were incubated at 22 °C overnight in the dark.

Observation of GFP fluorescence

Fluorescence imaging of protoplasts expressing SrAOX-GFP, SrUCPA-GFP, and SrNDA1-GFP was performed using a confocal microscope (LSM700; Zeiss). GFP was visualized by excitation with a laser at 488 nm and a detector set to less than 550 nm for emission. Transfection efficiency of the protoplasts was estimated by calculating the ratio of cells expressing GFP proteins in total number of cells.

Observation of MitoTracker

Protoplasts expressing SrAOX-GFP, SrUCPA-GFP, and SrNDA1-GFP were incubated in 100 mL WI solution containing 10 mM MitoTracker at 28 °C for 5 min. After a brief centrifugation for 10 s, precipitated protoplasts were resuspended in 100 mL WI solution and centrifuged for 30 s. The precipitated protoplasts were resuspended in 50 mL WI solution, stained with MitoTracker, and observed less than 30 min after staining by fluorescence imaging using a confocal microscope (LSM700; Zeiss). The red fluorescence of MitoTracker was visualized by excitation with a laser at 555 nm and a spectral detector set to more than 560 nm for emission.

Results

Isolation of intact protoplasts from skunk cabbage leaves

Protoplasts were isolated from skunk cabbage leaves according to the protoplast preparation from Arabidopsis leaves (Yoo et al. 2007; Zhai et al. 2009) (Fig. 1). For leaf-derived protoplasts from potted skunk cabbage plants, the whorled leaves that developed in the blooming season (reproductive stage) were cut off (Fig. 1a) and several inner leaves (young leaves) were selected (Fig. 1b); the outer leaves were too hard for protoplast isolation. Alternatively, young leaves in the central portion of a plant with developed leaves were used (Fig. 1c). The young leaves were sliced with a razor blade and degraded in a buffer containing macerozyme and cellulase to release the protoplast (Fig. 1d). The released protoplasts were purified by sucrose density gradient centrifugation and collected at the interface of the enzyme and W5 solution (Fig. 1e). The sucrose density gradient centrifugation procedure is an essential step in the high-purity protoplast isolation from the leaves of potted skunk cabbage plants, as indicated by a large number of needle-like structures, possibly crystals of calcium oxalate, present in the solution when this step was omitted (Fig. 1f). The implemented procedure yielded 4.14×10^5 protoplasts with high purity from 1 g of young leaves (Fig. 1g and Table S2).

After protoplast preparation from potted skunk cabbage plants, the shoot apex was excised from 69 skunk cabbage plants to prepare shoot-tip culture systems (Fig. 2 and Table S3). Among those, 15 individuals survived for more than one year, and 6 of the 15 individuals possessed a high ability to form multiple shoots. Of the 6 individuals, only two individuals survived for more than 5 years, and these had an outstanding ability to propagate from one shoot into more than 50 shoots (Fig. 2a). We used these plants for protoplast preparation from shoot tip-cultured plants. Interestingly, some plants were able to flower within a year (Fig. 2b), although those individuals were unable to form multiple shoots.

For protoplast isolated from shoot tip-cultured skunk cabbage plants, the plants propagated from a shoot tip (Fig. 2c) were continuously cultured until the shoots elongated and 2-3 leaves were developed (Fig. 2d). Their well-developed leaves were selected and sliced with a razor blade. Sliced leaves were subjected to protoplast isolation following the procedure for leaf-derived protoplast preparation from potted plants. The greenish band derived from protoplast fractions was collected at the interface of the enzyme and W5 solution (Fig. 2e). This procedure yielded 3.95×10^5 high-purity protoplasts from 1 g of leaves (Fig. 2f and Table S2). Compared with the protoplasts derived from potted plants, protoplasts from shoot tip-cultured plants contained a larger amount of chloroplasts, but their size was slightly smaller.

High-purity protoplasts were successfully isolated from both potted and shoot tip-cultured skunk cabbage plants. Shoot tip-cultured systems are suitable for year-round experimentation compared with potted plants, which are less likely to survive during the summer season under laboratory conditions.

Establishing an efficient transient gene expression system in skunk cabbage protoplasts

We developed an efficient transfection procedure for the protoplasts using fluorescence microscopy (Axioimager A2; Zeiss) (Fig. 3). Leaf-derived skunk cabbage protoplasts from potted plants were transfected with *35S::GFP* using a PEG-calcium-mediated transfection method. The fluorescent microscopy results revealed that approximately 50% of the transfected protoplasts expressed nuclear-targeted GFP, suggesting a high transfection rate (Fig. 3a). The protoplasts not transfected with the vector showed no fluorescent signal. Transfection efficiency tended to decrease when more than 10 mg of DNA or 2.0×10^5 cells were used in the

transfection procedure (Fig. 3b). The transfection procedure was optimized on the basis of these results, although there was no statistically significant difference ($p>0.05$) in transfection efficiency between procedures with different amounts of DNA or cell number (Fig. 3b). These results clearly indicate that skunk cabbage leaf protoplasts were efficiently transfected with exogenous DNA. Transfection efficiency of the protoplasts from shoot tip-cultured plants was similar to that of the protoplasts from potted plants (Fig. S1).

Localization analyses of mitochondrial energy-dissipating proteins using skunk cabbage leaf protoplasts

Mitochondrial energy-dissipating systems via AOX, UCP, and type II NAD(P)H dehydrogenase are proposed to be involved in floral thermogenesis because these proteins dissipate or decrease proton gradient via the inner mitochondrial membrane, leading to heat generation. In addition to the theoretical considerations, *SrAOX* and *SrNDA* mRNAs were specifically expressed in heat-producing tissues of skunk cabbage, and the expression levels of the SrUCPA protein in thermogenic tissues or stages were higher than those in non-thermogenic ones (Ito-Inaba et al. 2008a; Kakizaki et al. 2012). To develop cell experimental systems expressing these proteins that can be utilized to examine their roles in floral thermogenesis, we analyzed the localization of SrAOX, SrUCPA, and SrNDA1 proteins using transient gene expression systems in leaf protoplasts from potted plants (Fig. 4) and shoot tip-cultured plants (Fig. 5). The complete sequence of these three proteins without a stop codon was fused to the GFP protein, and the sequence encoding the GFP fusion protein was placed downstream of the cauliflower mosaic virus *35S* promoter. The constructs *35S::SrAOX-GFP*, *35S::SrUCPA-GFP*, and *35S::SrNDA1-GFP* were transiently expressed in leaf protoplasts from both potted and shoot tip-cultured plants, which was confirmed by GFP fluorescence observed by confocal laser microscopy.

In leaf protoplasts from potted plants (Fig. 4), SrAOX-GFP and SrNDA1-GFP exhibited similar green fluorescent patterns localized to the mitochondria, which were stained by MitoTracker. Green fluorescence in protoplasts expressing SrUCPA-GFP was observed as enlarged particles that were much larger than those observed in protoplasts expressing other GFP constructs. In the control protoplast expressing GFP protein, green fluorescence was localized mainly to the nucleus and cytosol, and the mitochondria were stained with MitoTracker.

In the leaf protoplast from shoot tip-cultured plants (Fig. 5), the patterns of green fluorescence observed in protoplasts expressing SrAOX-GFP, SrNDA1-GFP, and GFP were almost the same as that in the leaf protoplasts from potted plants. However, we additionally observed a clear pattern of autofluorescence of the chloroplasts that were abundant in the leaf protoplasts from shoot tip-cultured plants (Fig. 2f). Although MitoTracker staining was unsuccessful in those protoplasts, the size and morphology of the observed green fluorescence were quite similar to those of the mitochondria observed in potted plants (Fig. 4). In the protoplasts expressing SrUCPA-GFP, a slightly different pattern (a weak and obscure signal) of green fluorescence was observed, and these protoplasts were fragile for unknown reasons.

Discussion

The most common source for protoplast isolation is leaf tissue. Protoplast has been successfully isolated from the plant mesophyll of Arabidopsis (Yoo et al. 2007; Zhai et al. 2009), tobacco (Nagata and Takebe 1971), maize (Cao, et al. 2014), rice (Page et al. 2019; Zhang et al. 2011), and other non-model plant species,

such as the legumes *Medicago sativa* (Song et al. 1990) and *Phaseolusvulgaris* (Nanjareddy et al. 2016)), switchgrass (*Panicum virgatum)* (Burris et al. 2016), oil palm (*Elaeis guineensis*) (Masani et al. 2014), rubber tree (*Hevea brasiliensis*) (Zhang et al. 2016), and *Magnolia* (Shen, et al. 2017). The protoplast isolation efficiency exceeds 1.0×10^6/g fresh weight (FW) in well-studied plants; it is 3.0×10^7 in Arabidospsis (Zhai et al. 2009), 1-5 × 10^6/FW of ca. 10 leaves of maize (Cao et al. 2014), 1.0×10^7 /ca. 40-60 seedlings in rice (Zhang et al. 2011), and 3.5×10^7 /g FW in orchid (Ren et al. 2020). Although the protoplast yield from young skunk cabbage leaves is one order of magnitude lower than that from young or mature leaves reported in other plants, it was sufficient for the protein localization study (Figs. 4 and 5). Flower petals have been selected as an alternative source for protoplast isolation in ornamental plants such as rose (*Rosa* 'Yves Piaget') (Hirata et al. 2012) and the orchids *Cymbidium* (Ren et al. 2020) and *Phalaenopsis* (Lin et al. 2018). Given that the protoplasts isolated from different plant organs or tissues maintain their cellular identity (Hirata et al. 2012), we conclude that the protoplasts from thermogenic organs or tissues are suitable for functional characterization of thermogenesis-related genes in aroids and other thermogenic plants. However, we were unable to isolate protoplasts from spadices (flowers in a broad sense) of skunk cabbage, possibly due to the hardness of petal tissues in this plant.

The PTE system is an important experimental tool in plant molecular biology. Protoplast transfection efficiency >50% is required for reliable and reproducible experimental data (Yoo et al. 2007). In our study, after the removal of a large amount of needle-like compounds, which seem to break intact protoplasts, by the sucrose density gradient step, the transfection efficiency of skunk cabbage leaf protoplasts was increased to approximately 50%, indicating that our system meets the criteria of the model plant study. However, more efficient protoplast isolation and transfection protocols should be established for future studies on gene silencing, protein-protein interaction, and reporter assays.

Protein localization using mitochondrial energy-dissipating proteins SrAOX, SrUCPA, and SrNDA1 fused to GFP revealed that the fluorescence signal of these fusion proteins overlapped with the MitoTracker-stained organelle. These results indicate that the transient expression system in skunk cabbage protoplasts is a useful tool for analyzing protein localization using target proteins fused to GFP. It should be noted that the signal of SrUCPA-GFP is quite different from that of SrAOX-GFP and SrNDA1-GFP. However, considering that SrAOX and SrUCPA are localized in the inner mitochondrial membrane (Ito-Inaba et al. 2008a), it is reasonable to assume that SrUCPA-GFP proteins were targeted to the mitochondria. Possibly, excess uncoupling in mitochondria due to accumulated UCP causes serious damage to mitochondrial function (e.g., fusion-fission events), and results in aggregation of damaged mitochondria.

Isolation of protoplasts with high yield from tissues other than leaves in skunk cabbage remains challenging. A successful isolation requires careful consideration of the species type, source materials, enzyme combinations, and sugar concentration regulating osmotic pressure. These parameters have been difficult to thoroughly examine in skunk cabbage because the plant material is not readily available due to their limited habitat distribution and restricted blooming season. Similar difficulties may be encountered with many other thermogenic plants. However, such limitations in these plants can be overcome by utilizing the regeneration ability of the plants, as demonstrated in the shoot tip-culture system implemented in this study. The survival rate of shoot tip-cultured skunk cabbage plants seems to largely depend on the vitality of individual plants and the cutting technique, which should preserve the shoot apex intact. Once a shoot apex has developed into a

seedling, in which the shoot reached 3-5 cm in height, this individual is able to survive on MS-based media for more than one year in most cases. However, in the present systems, none of the shoot tip-cultured plants were able to survive in soil conditions, even if the plants were well developed (Fig. 2b). Therefore, it is necessary to identify the optimal conditions under which shoot tip-cultured plants grown on MS-based media will be able to acclimatize to soil conditions. Furthermore, it should be noted that in this study, the mother plants used for tip-cultured plants were originally grown as potted plants and were used in various experiments, including protoplast isolation, in our laboratory. Possibly, this is the reason for low survival rate of shoot-tip cultured plants (Table S3). The survival rate of them will increase if more fresh or younger plants were used as mother plants for tip-cultured plants.

Declarations

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Conflicts of interest:
The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability

Not applicable

Authors' contributions

HM, MO, MK, DK, TI, and YII conducted the experiments. YII and TI analyzed the data. YII contributed to the experimental design and wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

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Tables

Table 1. Aroids (Araceae) with known thermogenic ability in their flowers.

Genus	Botanical name	Part	Temperature (°C)[a]			Ref.[c]
			T_{flower}	T_{air}	T_{flower}-T_{air}[b]	
Alocasia	*A. macrorrhizos*	Appendix	47.4	26.0	21.4	(1)
	A. odora	Appendix	≈30.5	≈26.5	≈4	(2)
		Spadix (Staminate part)	≈29.5	≈26.0	≈3.5	(2)
Anubias	*A. afzelii*	Spadix	27.8	22.2	5.6	(3)
	Anubias sp	Spadix	26.4	21.7	4.7	(3)
	A. barteri	Spadix	25.3	23.9	1.4	(3)
Amorphophallus	*A. konjac*	Appendix	≈32	≈29	≈3.0	(4)
	A. paeoniifolius/campanulatus[d]	Appendix	≈31	≈24.5	≈6.5	(5)
	A. titanum	Appendix	36.6	24	12.6	(4)
Arum	*A. concinnatum*	Appendix	33.2	22.1	11.1	(6)
		Male florets	26.2	21.4	4.8	(6)
	A. dioscoridis[d]	Spadix	≈40	≈22.5	17.5	(5)
	A. italicum	Spadix	31.4	20.6	10.8	(6)
	A. maculatum	Appendix	≈34.0	≈24.4	9.6	(7)
Cercestis	*C. stigmaticus*	Spadix	30.1	27.6	2.5	(3)
Colocasia	*C. esculenta*	Appendix	≈29.1	≈22.3	6.8	(8)
		Male portion	-	-	7.4	(8)
	C. gigantea	Male part	≈42.2	25.6	16.6	(9)
Culcasia	*C. saxatilis*	Spadix	22.7	20.2	2.5	(3)
	Culcasia sp	Spadix	25.2	23.6	1.6	(3)
Sauromatum	*S. guttatum*[c]	Appendix	32	20	12	(10)
Philodendron	*P. acutatum*	Spadix	36.9	23.5	≈13.4	(3)
	P. appendiculatum	Spadix	27.1	22.9	4.2	(6)
	P. grandifolium	Spadix	22.6	16.3	6.3	(6)
	P. imbe	Spadix	21.9	12.9	9	(6)

	P. melinonii	Spadix	39.5	27.2	12.3	(6)
	P. pedatum	Spadix	34.6	22.8	11.8	(3)
	P. selloum	Spadix	40.3	18.5	21.8	(6)
	P. solimoesense	Spadix	41.1	27.5	13.6	(6)
Helicodiceros	*H. muscivorus*	Appendix	29.8	15.2	14.6	(6)
Dracunculus	*D. vulgaris*	Appendix	26.6	24.1	2.5	(6)
		Male florets	18.6	10.3	8.3	(6)
Symplocarpus	*S. foetidus*	Spadix	18.2	-7.4	25.6	(6)
	S. renifolius	Spadix	23.4	-0.2	23.6	This study [e]
	S. nabekuraensis	Spadix	21.6	16.5	5.1	(11)
Montrichardia	*M. aborescens*	Spadix	35.5	24.2	11.3	(3)
Homalomena	*H. megalophylla*	Spadix (Staminate part)	≈38	≈24	≈14	(12)
	H. pendula	Spadix (Staminate part)	≈38.4	≈23.0	≈15.4	(12)
	H. propinqua	Spadix	-	-	2.3	(13)
	H. rubescens	Spadix	31.8	21.3	10.5	(3)

[a] If the temperatures were not provided in previous reports, approximate values were inferred from graphs

Figures

Fig. 1

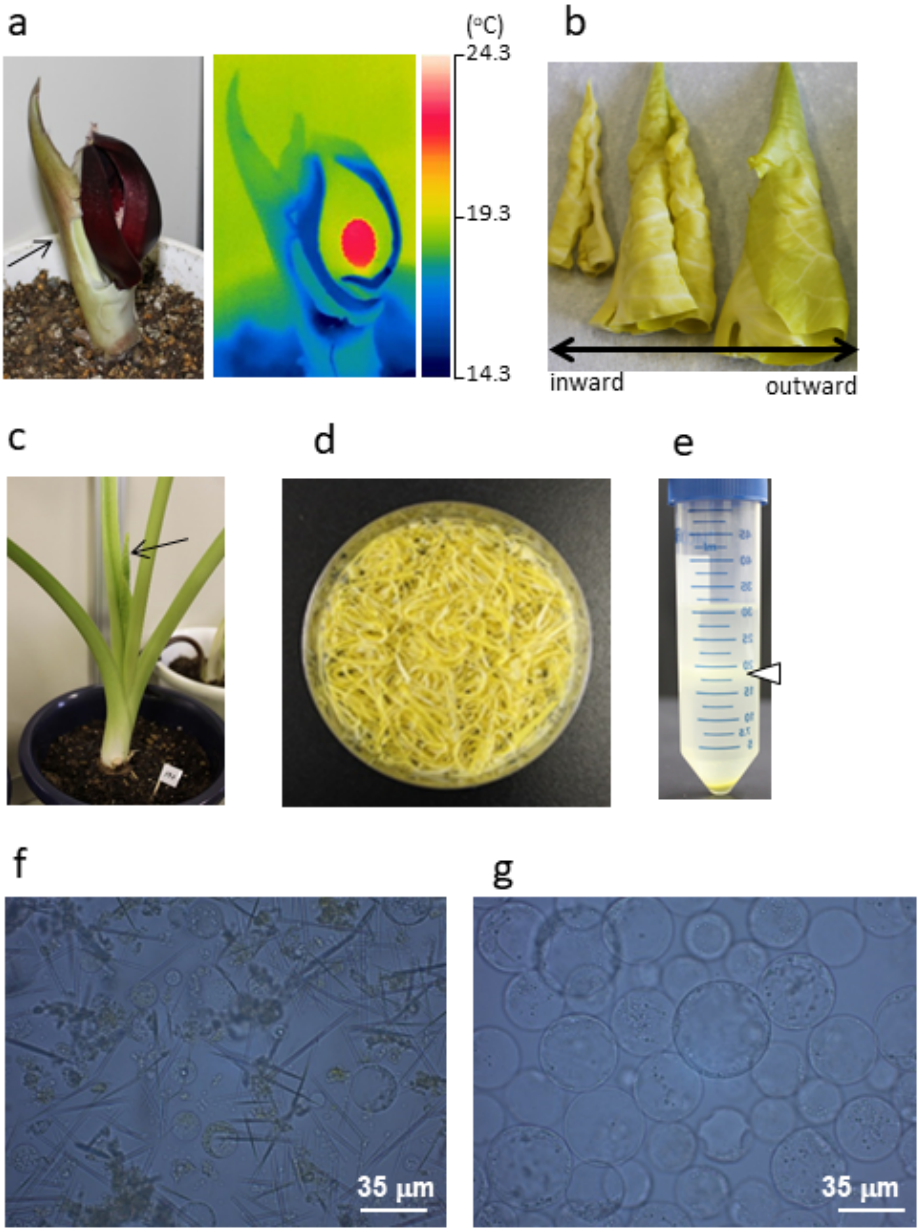

Figure 1

Protoplast isolation from the leaves of potted skunk cabbage plants a Potted skunk cabbage at the blooming stage. Arrow shows the leaf, with inner leaves that are suitable for protoplast isolation. Thermal images (right) were obtained using an FLIR SC620 thermal imager (FLIR Systems). b Skunk cabbage leaves cut from a potted plant (a). Potted plants have many layers of whorled leaves. The inner leaves are younger and softer. c Potted skunk cabbage with developed leaves at the post-blooming stage. Arrow shows the young leaf that appeared from the central portion of the plant. d The inward young leaves, sliced with a razor blade, were soaked in enzyme solution. e After sucrose density gradient centrifugation, the protoplasts were collected at the interface of W5 buffer and 21% sucrose solution (white arrow). f Protoplasts isolated from (e). A large number of needle-like compounds, possibly derived from calcium oxalate, appear with the protoplasts when

the sucrose density gradient centrifugation step is omitted. g Protoplasts isolated from (e). Protoplasts after implementing a sucrose density gradient centrifugation

Fig. 2

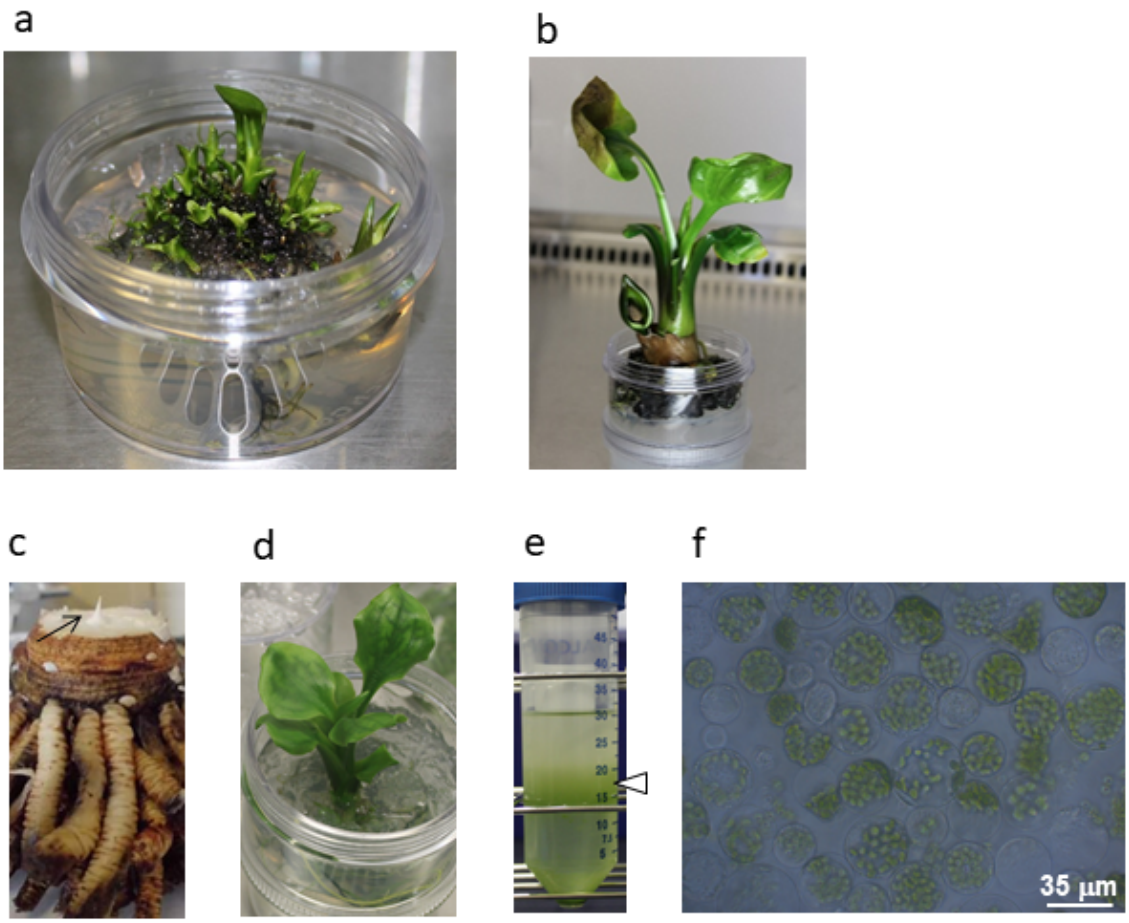

Figure 2

Protoplast isolation from the leaves of tip-cultured skunk cabbage plants a Multiple shoots appeared from the shoot apex. b A shoot-tip cultured plant that successfully flowered. c Shoot apex (indicated by arrow) before being cut out. d Shoot-tip cultured skunk cabbage. The developed leaves were used for protoplast isolation. e Protoplasts collected following the same protocol as that in Fig. 1e. f Protoplasts isolated from (e)

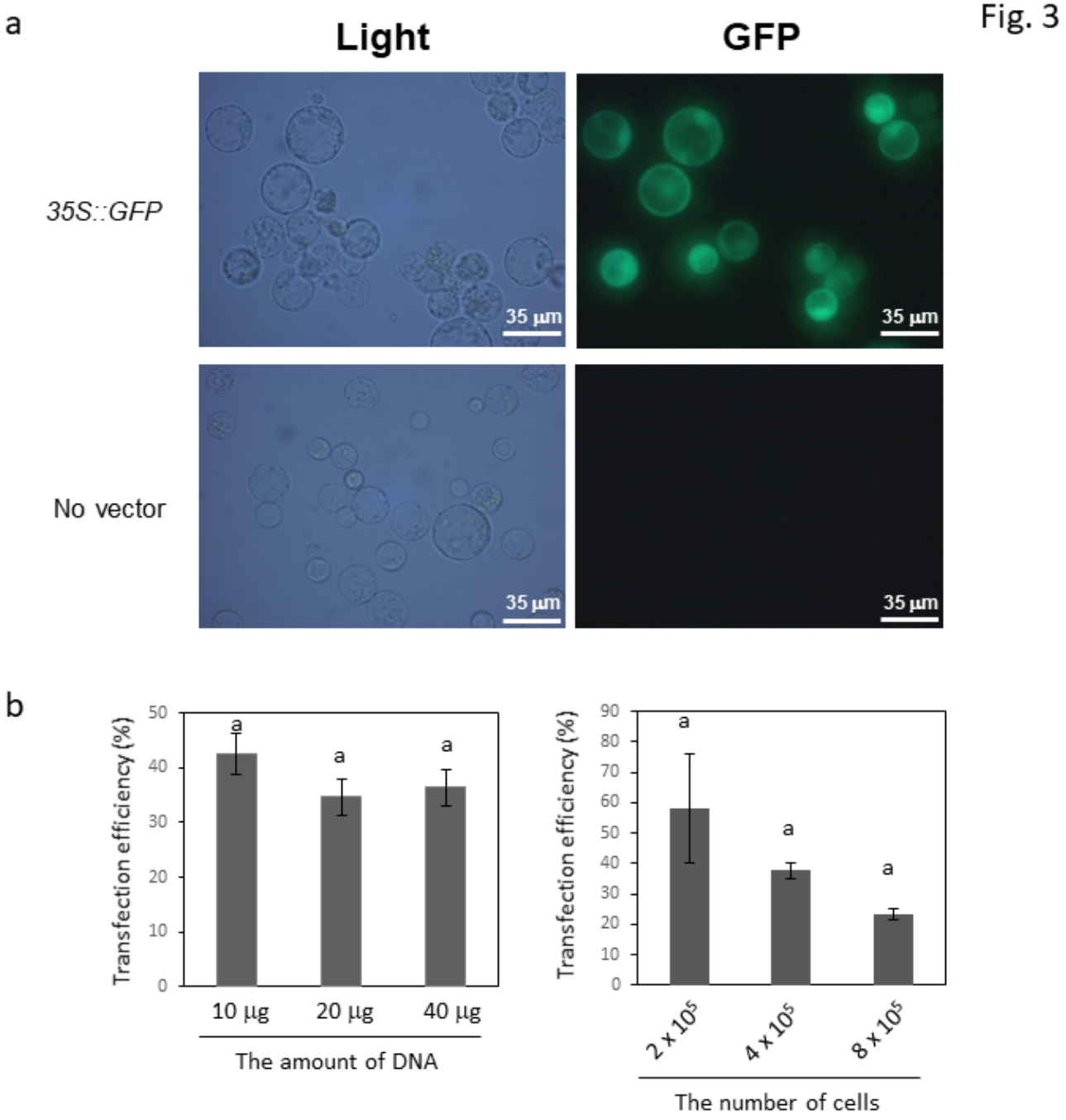

Figure 3

Transfection of leaf-derived protoplasts, which were isolated from leaves of potted plants, with 35S::GFP a Bright-field (Light) and fluorescent microphotographs (GFP) were collected on the Zeiss AxioImager A2 microscope equipped with appropriate filter sets. b The transfection efficiency of skunk cabbage leaf protoplasts. Protoplasts were transfected with different amounts of DNA containing 35S::GFP (left). The transfection efficiencies were calculated and expressed as the mean ± SE for 10 fields of view for each DNA amount used. Different number of protoplasts was transfected with 10 µg of DNA containing 35S::GFP (right). The transfection efficiencies were calculated and expressed as the mean ± SE for 21 fields of view when 2 × 105 cells were transfected, 31 fields of view when 4 × 105 cells were transfected, and 14 fields of view when 8

× 105 cells were transfected, respectively. Both data (left and right) were analyzed by Tukey-Kramer multiple comparison test, which revealed no statistically significant differences between the different transfections

Fig. 4

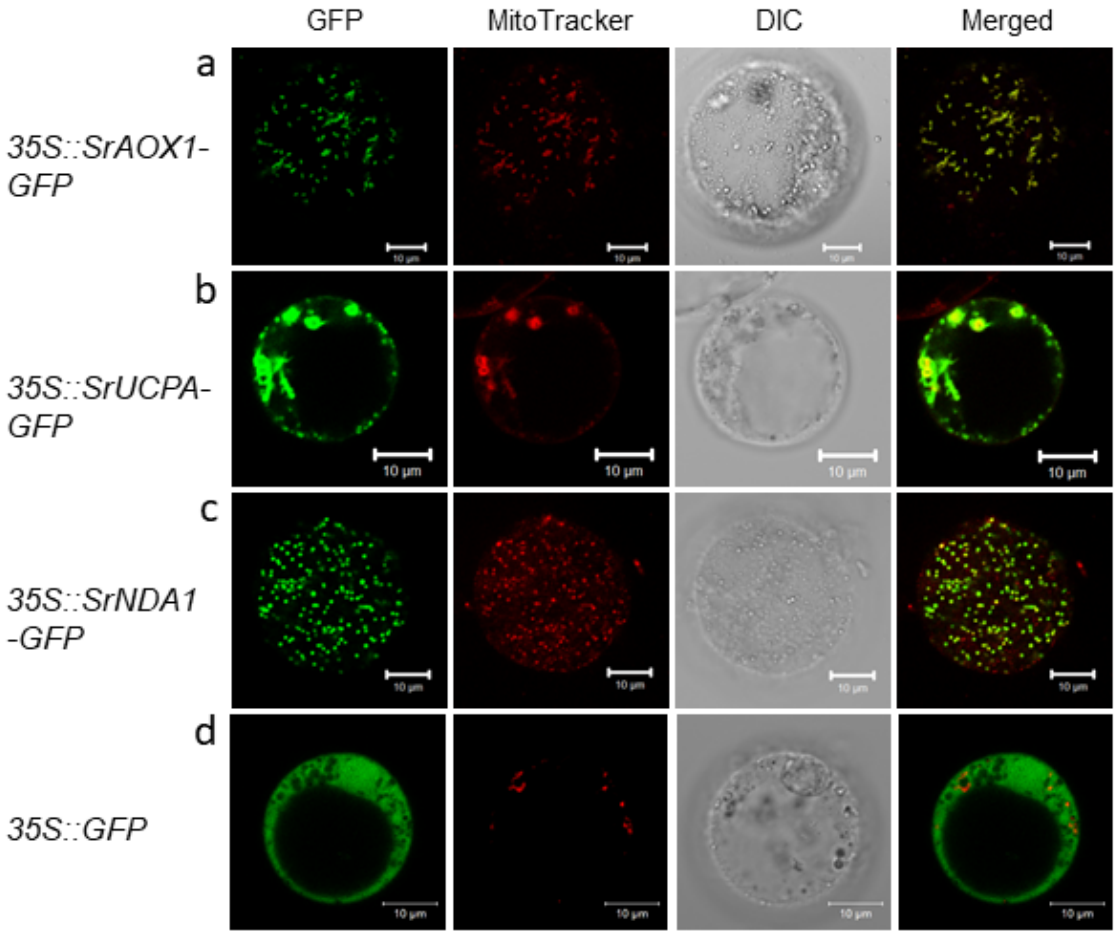

Figure 4

Subcellular localization of three energy-dissipating proteins, SrAOX, SrUCPA, and SrNDA1, fused to GFP and mitochondrial marker in leaf protoplasts isolated from potted skunk cabbage plants. SrAOX-GFP (a), SrUCPA-GFP (b), SrNDA1-GFP (c), and GFP proteins (d) were transiently expressed in these protoplasts. Confocal microscopy images show GFP fusion protein (GFP), mitochondria stained with MitoTracker (MitoTracker), differential interference contrast (DIC), and the overlay of the two channels (Merged). Bar in a-d: 10 µm

Fig. 5

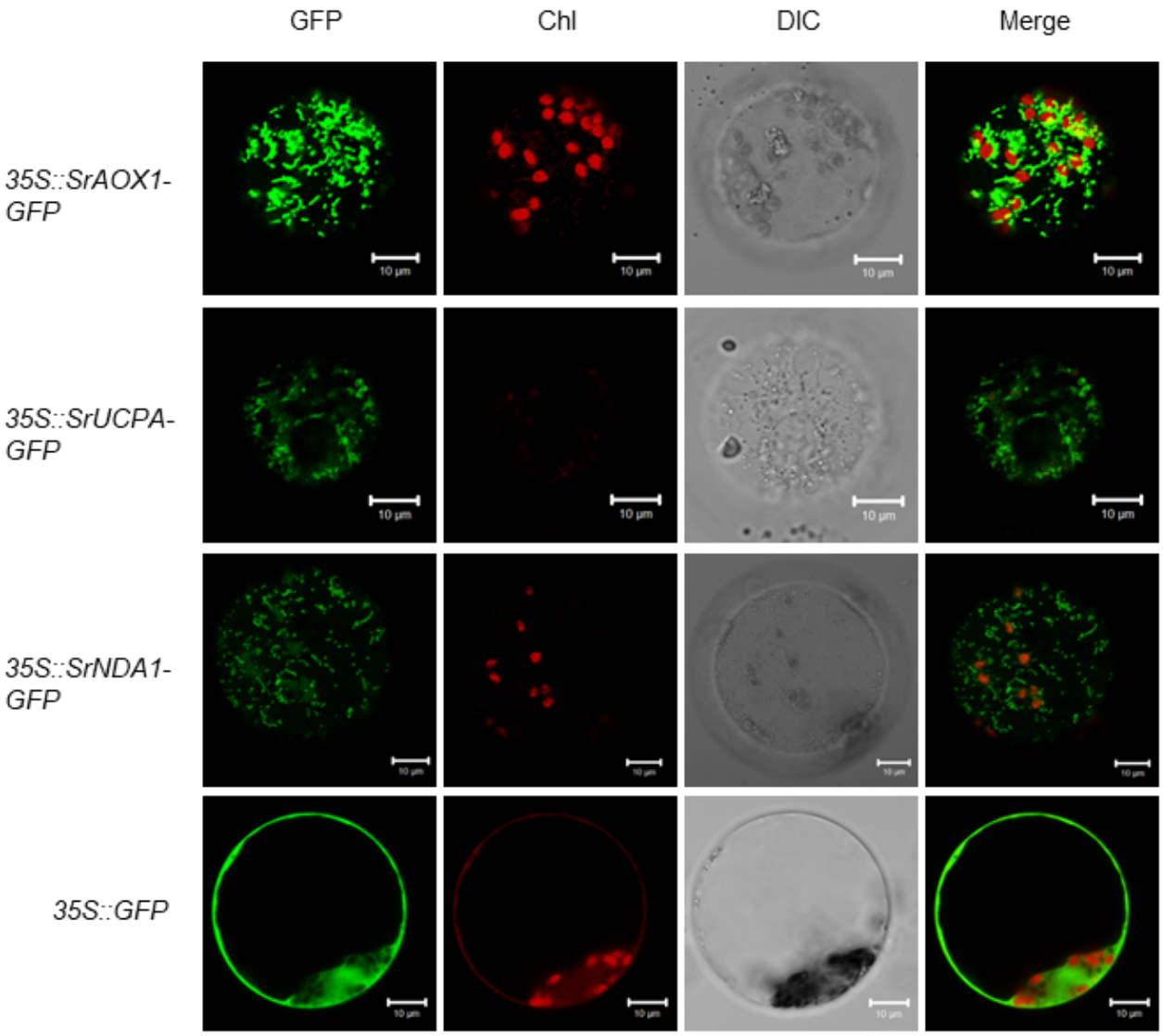

Figure 5

Subcellular localization of three energy-dissipating proteins, SrAOX, SrUCPA, and SrNDA1, fused to GFP and mitochondrial marker in leaf protoplasts isolated from shoot-tip cultured skunk cabbage plants SrAOX-GFP, SrUCPA-GFP, SrNDA1-GFP, and GFP proteins were transiently expressed in these protoplasts. Confocal microscopy images show GFP fusion protein (GFP), chloroplast autofluorescence (Chl), differential interference contrast (DIC), and the overlay of the two channels (Merged). Bar in a-d: 10 µm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1Maekawa.pptx
- SupplementaryTablesMaekawa.xlsx